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In the past year, I have made substantial progress towards completion of the tasks outlined in my Statemtn of Work. I have established a variety of stable cell lines, conducted preliminary purifications, and tested the functions of the BRCA1/BARD1 complex in *in vitro* assays for ubiquitination and transcription.

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Intro

In the past year, I have made substantial progress towards completion of the tasks outlined in my Statement of Work. I have established a variety of stable cell lines, conducted preliminary purifications, and tested the functions of the BRCA1/BARD1 complex in *in vitro* assays for ubiquitination and transcription.

Body

Task 1: To purify and characterize BRCA1 complexes from cultured mammalian cell lines.

A) Subcloning to assemble a retroviral zz-TEV fusion vector.
B) Infection and selection of stable cell lines
C) Purification by chromatography and affinity steps.
D) Identification of protein subunits by mass spectrometry and comparison between breast and non-breast cell line.

As anticipated, stable expression of BRCA1 is difficult, probably due to the growth suppressive properties of this protein. In addition, retroviral packaging of especially large constructs, like BRCA1, is inefficient, and yields low titers of virus. To address these identified problems, I adopted two alternative strategies. First, I used the *Flp-In* system (Invitrogen) to make tetracycline-inducible stable cell lines. This system uses site-specific recombination to generate stable cell lines with expression from a transcriptionally active genomic locus. The 293 cells were selected and maintained in the absence of BRCA1 expression, and, following tetracycline treatment, high levels of BRCA1 expression are induced (Figure 1). Second, taking advantage of the SV40 origin in the pBabe plasmid, I created a 293T cell line that stably expresses BRCA1 at near endogenous levels (Figure 2). The 293T cells most likely maintain the expression vector episomally, since 293T cells express T-antigen and replicate SV40 origin-containing plasmids like pBabe.

Using these cell lines, I have attempted several small scale purifications. Elution of the BRCA1 from IgG beads has not been possible by TEV cleavage as planned. This could be due to associated proteins blocking protease access to the TEV consensus sequence. Other methods of elution (acid, boiling) have released proteins which bind non-specifically to the IgG beads, obscuring any specific interactions. Interestingly, pre-treatment of the cells with a DNA damage agent allows efficient TEV protease elution of the purified BRCA1, as discussed further under Task 2.

As a complementary approach to purifications via affinity-tagged BRCA1, I have generated stable cell lines expressing proteins thought to interact with BRCA1, in hopes of purifying these complexes. I have established cell lines expressing components of the RNA Polymerase II (Pol II) core enzyme and holoenzyme (RPB8, RPB9 and CDK8) as well as the HMMR protein, which was identified as a BRCA1-interacting protein by yeast two hybrid analysis, and is also implicated in breast cancer (unpublished observations). Purification of these complexes is currently underway.

Task 2: To describe the dynamics of BRCA1 complex formation and redistribution.

A) Purify BRCA1 complexes from cells synchronized at different points in the cell cycle or following DNA damage.
B) Compare distribution of complexes by Native Blue PAGE.

To examine the effect of DNA damage on BRCA1 complexes I have conducted preliminary experiments with hydroxyurea (HU). HU depletes dNTP pools, leading to replication fork stalling and DNA damage. In the 293T cells which stably express zz-TEV-BRCA1, one hour pre-treatment of cells with HU led to the appearance of a higher molecular weight band above the BRCA1 band that may correspond to a phosphorylated form of BRCA1. Following HU treatment and purification on IgG beads, the BRCA1 is efficiently eluted by TEV cleavage (Figure 3). This suggests that HU treatment alters the context of the BRCA1 to allow TEV protease to access the cleavage site, perhaps by rearrangement of the protein complex that BRCA1 resides in. Prior work in the Parvin lab demonstrated that HU induces a shift from a larger BRCA1 complex to a smaller complex of unknown composition [1]. One candidate for this complex is the simple BRCA1/BARD1 heterodimer. By scaling up my purification, I hope to determine what the identity of the HU-induced complex is.

I have also spent time developing Native Blue PAGE as a method for separating mixtures of complexes. This technique was developed for separating the membrane-associated complexes of the mitochondria, but can be adapted for soluble protein complexes. While I have not yet had the opportunity to test this protocol on BRCA1 complexes, I have used the RPB9 stable cell line to purify Pol II complexes and demonstrated that the Pol II large subunit (RPB1) is distributed among a range of molecular weights, as expected given the variety of Pol II holoenzyme complexes in the cell (Figure 4). This preliminary work validates the utility of Native Blue PAGE for separation of macromolecular complexes.

Task 3: To test the function of purified BRCA1 complexes by *in vitro* assays.

While purification attempts have been ongoing, I have been working to establish and refine *in vitro* assays for BRCA1 function. For the time being, I have focused on the BRCA1/BARD1 heterodimer. This complex can be produced in insect cells by baculoviral infection, and we have generated a panel of BRCA1 mutant/truncation constructs for expression. I have tested these complexes in two assays relating to the BRCA1/BARD1 association with Pol II. The first assay uses the 12 subunit Pol II complex as a substrate for BRCA1/BARD1 ubiquitination. Prior work has shown that Pol II becomes ubiquitinated following DNA damage, and since BRCA1/BARD1 associate with Pol II, we hypothesized that BRCA1/BARD1 may target Pol II [2]. We have shown that BRCA1/BARD1, in the presence of E1 and E2 enzymes and ubiquitin, ubiquitinates the RPB1 subunit of Pol II (Figure 5). Additionally, using kinases specific for serine 2 or 5 in the RPB1 C-terminal domain (CTD) heptad repeats, we demonstrated that BRCA1/BARD1 has a preference for serine 5 phosphorylated CTD (Figure 6). We were able to confirm these *in vitro* results *in vivo*, conducting transfection experiments in tissue culture cell lines to demonstrate the same preference for serine 5 phosphorylated Pol II (Figure 7). In these experiments, the ubiquitination of Pol II was stimulated both by UV

irradiation of the cells and by overexpression of BRCA1. Interestingly, the serine 5 phosphorylated form of Pol II corresponds to a transcriptional promoter clearance/pre-elongation state, so our results are not strictly consistent with a model where BRCA1 ubiquitinates the form of Pol II expected to encounter DNA lesions. This complicates a model where BRCA1/BARD1 plays a role in transcription-coupled repair. However, since DNA damage leads to an alteration in Pol II phosphorylation state (unpublished observation), it is possible that serine 5 phosphorylation may not correspond to promoter clearance in that context [3].

One of the first functions associated with BRCA1 was transcriptional activation, and a range of p53-dependent and independent targets have been identified by reporter assays and DNA microarray studies [4] [5]. Thus, one way in which BRCA1 may function as a tumor suppressor is by upregulation of growth suppressive targets. The mechanism by which BRCA1 activates transcription remains unknown, and is not resolvable *in vivo* in by reporter assay. Using a fully purified *in vitro* transcription system, I have measured robust transcriptional activation by BRCA1/BARD1. This assay is the first demonstration of transcriptional activity *in vitro* by full length BRCA1 and is the starting point for a detailed mechanistic investigation. The minimal set of factors required for BRCA1 transcriptional activation are TFIIA, TFIIB, TBP, TFIIF and the core Pol II complex. The ratio of transcriptional activation is further enhanced by the addition of TFIIE and TFIIH (Figure 8). BARD1 is dispensable, and I am currently working to determine which domains of BRCA1 are required. Preliminary results indicate that the amino-terminus is required, which is surprising given that prior work has implicated the carboxy-terminus, both *in vivo* by reporter assay, and *in vitro* by Gal4 fusion [6]. Also, early experimental results indicate that BRCA1 exerts its effects during initiation.

In future experiments I plan to conduct gel shift experiments using BRCA1 and the transcription factors to determine how BRCA1 might affect assembly of a pre-initiation complex onto template DNA. I am also interested in determining whether mutations in the amino-terminus of BRCA1, identified for disruption of ubiquitination activity, also abolish transcriptional function.

Key Research Accomplishments

• Established cell lines for affinity purification of BRCA1 complexes (completed).

• Identified alterations in BRCA1 following HU treatment that may correspond to dynamic changes in complexes (in progress).

• Characterized the *in vitro* ubiquitination of Pol II by BRCA1/BARD1; confirmed the results *in vivo* (completed).

• Developed an *in vitro* system to assay the transcriptional activity of BRCA1. Conducted initial experiments to determine the mechanism by which BRCA1 activates transcription. (in progress).

Reportable Outcomes

1) Stable cell lines expressing affinity-tagged BRCA1, Pol II components, and HMMR.

2) BRCA1/BARD1 ubiquitinate phosphorylated RNA polymerase II. Lea M. Starita*, Andrew A. Horwitz*, Michael-Christopher Keogh, Chikashi Ishioka, Jeffrey D. Parvin, Natsuko Chiba. Submitted. **These authors contributed equally.*

Conclusions

The studies described here are aimed at understanding how BRCA1 functions as a tumor suppressor. I have made progress in several areas of my fellowship proposal. The work to date purifying BRCA1-containing complexes has identified a HU-induced alteration of BRCA1 that may correspond to dynamic changes in it protein-protein associations following DNA damage. BRCA1 has been attributed a wide variety of cellular functions, and the complex in which it resides most likely determines its function. Therefore, the identification of protein complexes induced by stimuli such as DNA damage is important for understanding how BRCA1 accomplishes tumor suppression. In future work, I hope to characterize the HU-induced complex, and to determine whether there is variation in this complex between breast and non-breast cell lines. Such differences could provide clues to the tissue-specificity of BRCA1 tumor suppression.

A second area of investigation in the past year has focused on BRCA1 complex function *in vitro*, in particular as it relates to the interaction between BRCA1/BARD1 and the Pol II complex. Regulation of Pol II by BRCA1/BARD1 could effect tumor suppression in two ways. First, in transcription-coupled repair, BRCA1/BARD1 might be responsible for the ubiquitination of Pol II which is known to follow DNA damage. We confirmed that Pol II was a substrate for BRCA1/BARD1 *in vitro* and *in vivo*. Second, BRCA1/BARD1 could act as an activator of transcription, targeting growth suppressive genes. Using a fully purified *in vitro* system, I have shown that BRCA1 activates transcription, working at the initiation stage. This purified system will be used to determine the mechanism by which BRCA1 activates transcription. In the future, I hope to link these *in vitro* studies to the ongoing BRCA1 complex purifications by testing purified complexes for activity in these assays.

Figure 1: Tetracycline-inducible expression of zzTEV-BRCA1 from *Flp-In* 293 stable cell line. Western blot of cell lysates with anti-BRCA1 antibody.

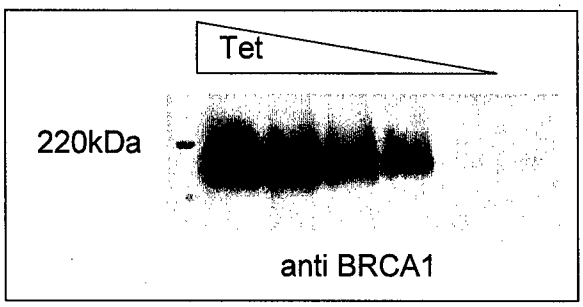


Figure 2: Stable expression of zzTEV-BRCA1 in 293T cells. zzTEV-BRCA1 was purified by IgG beads, using empty-vector stable 293T cells as a negative control. Western blots were probed with anti-BRCA1.

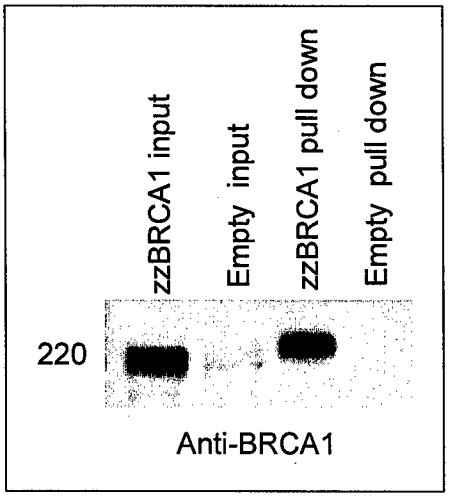


Figure 3: Elution of BRCA1 from IgG matrix by TEV protease cleavage. Following purification of zzTEV-BRCA1 from 293T stable cells, washed beads (B) or TEV eluates (E) were separated by SDS PAGE and silver stained. TEV elution was for 0, 1 or 4 hrs at 4 degrees.

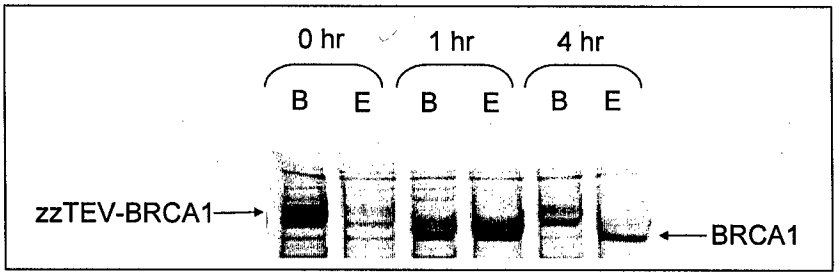


Figure 4: Native Blue PAGE example. Affinity purified RPB9 (Pol II) complexes were separated under native conditions in the first dimension, then under denaturing conditions in the second dimension. The gel was transferred and blotted with an antibody to the Pol II subunit RPB1.

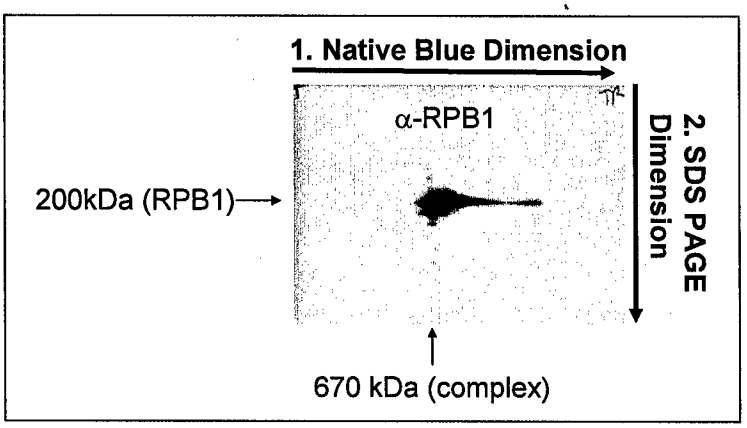


Figure 5: BRCA1/BARD1 ubiquitinates hyperphosphorylated Pol II, dependent on E1, E2 (UbcH5) and ubiquitin.

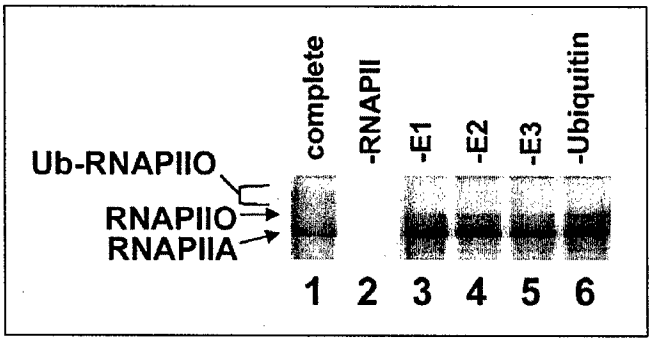


Figure 6: BRCA1/BARD1 ubiquitination is specific for the Serine 5 phosphorylated Pol II CTD.

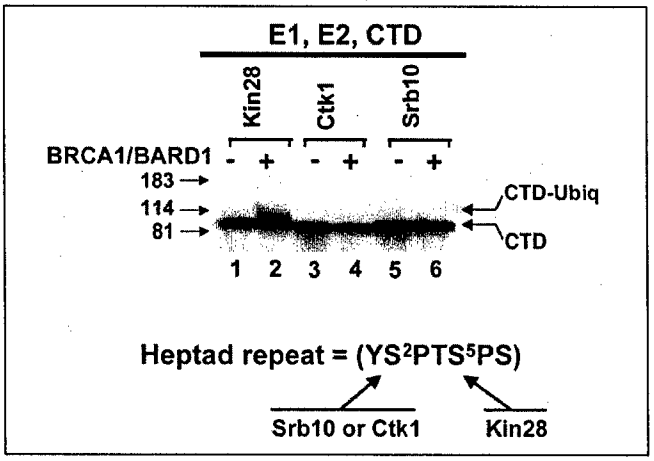


Figure 7: *in vivo* ubiquitination of Pol II is stimulated by BRCA1(ΔM) and UV irradiation. 293 cells were transfected with expression constructs for full length HA-BRCA1 or HA-BRCA1(ΔM), which has an internal deletion. Myc-tagged ubiquitin was co-transfected. The H14 antibody detects Serine 5 phosphorylated Pol II. The 8WG16 antibody detects unphosphorylated Pol II.

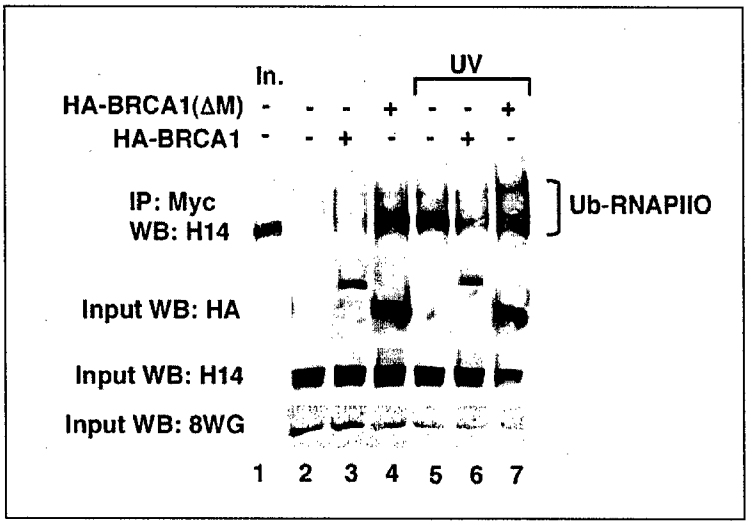
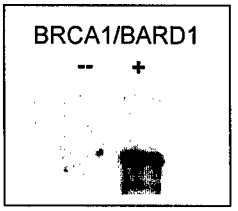


Figure 8: Activation of transcription by BRCA1/BARD1. *In vitro* transcriptions were performed using a G-less cassette template and a set of fully purified factors.



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